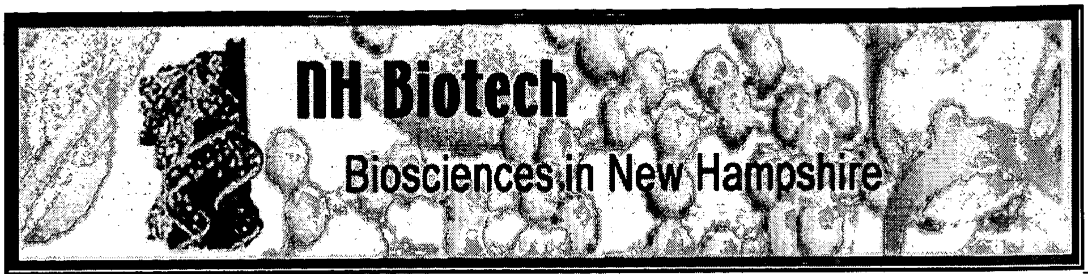

WebTracker

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- Ronni L. Sherman, Executive Vice President, General Counsel
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- Michael L. Muhich, Ph.D., Vice President, Operations

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	0130 840 911	0130 762 088	069 9509 6197
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	0800 023 0446	0800 023 0447	0800 023 0448
Switzerland	00800 7000 7000	00800 7001 7001	00800 7400 7400
	061 6930 540	061 6930 544	01 800 9045
	0800 830 250	0800 825 225	
United Kingdom	00800 7000 7000	00800 7001 7001	00800 7400 7400
	01223 420 955	01223 420 234	0171 365 1056
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Complete Control® Inducible Mammalian Expression System

Contents

Complete Control® Inducible Mammalian Expression System

Materials Provided

Material provided	Concentration	Quantity		
		Catalog #217460	Catalog #217461	Catalog #217468
pERV3 receptor vector	1 µg/µl	100 µg	—	100 µg
pEGSH expression vector	1 µg/µl	—	20 µg	20 µg
pEGSH-Luc vector (positive control)	1 µg/µl	—	20 µg	20 µg
pEGSH sequencing primer[a] (lyophilized)	—	—	2.5 µg	2.5 µg
T3 20-mer sequencing primer[b]	—	—	2.5 µg	2.5 µg
XL1-Blue host strain[c]	—	—	—	500 µl (glycerol stock)
Ponasterone A (lyophilized)[d]	—	—	—	1 mg

[a] pEGSH forward sequencing primer: 5′-CTCTGAATACTTTCAAAAGTTAC-3′.

[b] T3 promoter sequencing primer: 5′-AATTAACCCTCACTAAAGGG-3′.

[c] Genotype: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q} *Z*Δ*M15 Tn10* (Tet^r)].

[d] See *Preparation of Media and Reagents*.

Storage Conditions

XL1-Blue Host Strain: –80°C
All Other Materials: –20°C

Additional Materials Required

1× Phosphate-buffered saline (PBS) solution[§]
LB agar[§]
LB liquid medium[§]
Ampicillin
Cesium chloride
G418 sulfate or Geneticin® antibiotic
Hygromycin
Luciferase assay kit
Luminometer
Mammalian transfection solutions
Restriction enzymes
Plasmid DNA isolation solutions
T4 DNA ligase
10× Ligase buffer[§]
TE buffer[§]
10 mM rATP
100% (v/v) Ethanol

[§] See *Preparation of Media and Reagents*.
Revision #042006

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Stratagene has a license to sell the Complete Control Mammalian Expression System ("Expression Kit") to scientists for research purposes only, under the terms described below. Use of this system by commercial entities for any commercial purpose requires the user to obtain a commercial license as detailed below.

Patent Pending

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pERV3 Vector

Use of the translation enhancer of the pERV3 Vector and the Complete Control® vector kit is covered by U.S. Patent No. 4,937,190 and is limited to use solely for research purposes. Any other use of the translation enhancer of the pERV3 Vector and the Complete Control vector kit requires a license from WARF.

CMV Promoter

The use of the CMV Promoter is covered under U.S. Patent Nos. 5,168,062 and 5,385,839 owned by the University of Iowa Research Foundation and licensed FOR RESEARCH USE ONLY. For further information, please contact UIRF at 319-335-4546.

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INTRODUCTION

System Overview

Stratagene's Complete Control® inducible mammalian expression system is a gene transfer system that allows precise control of gene expression in a wide variety of mammalian cell types. Development of the Complete Control system is based upon the finding that the insect hormone ecdysone or its analog ponasterone A (ponA) can activate transcription in mammalian cells harboring both the gene for the *Drosophila melanogaster* ecdysone receptor and a promoter containing a binding site for the ecdysone receptor.[1]

The Complete Control system has several advantages over other inducible systems. PonA has no known measurable effect on mammalian physiology. PonA has a short in vivo half-life, and its lipophilic nature allows it to efficiently penetrate all tissues, including the brain. The result is rapid and potent induction of gene expression and rapid clearance. A 1000-fold induction of a reporter gene, with negligible basal expression, has been obtained with the Complete Control system.[2]

PREPARING THE HOST CELLS

Note *Immediately upon arrival, the vials should be stored at –20 or –80°C. Most strains remain viable longer if stored at –80°C. Avoid repeated freeze–thaw cycles of the host strain to maintain extended viability.*

Streaking the Host Cells

1. Scrape a few splinters of solid ice from the stored cells with a sterile wire loop.
2. Streak the splinters onto an LB agar plate (see *Preparation of Media and Reagents*).
3. Incubate the plates overnight at 37°C.
4. Store the plates at 4°C for up to 1 week, then restreak the colonies onto a fresh plate.

Preparing –80°C Bacterial Glycerol Stocks

1. In a sterile 50-ml conical tube, inoculate 10 ml of LB liquid medium[§] with one or two bacterial colonies from step 4 of *Streaking the Host Cells*. Incubate the cells at 37°C with vigorous agitation until the cells reach late log phase (OD_{600} = ~1.0).
2. Add 4.5 ml of a sterile glycerol–LB liquid medium solution (1:1) to the cells and mix well.
3. Aliquot the glycerol stock into sterile microcentrifuge tubes (1 ml/tube).

   The glycerol stocks can be stored at –20°C for 1–2 years or at –80°C for more than 2 years.

Note *These XL1-Blue cells may be made competent for later use in transformation.*[6]

Overview of the Complete Control® Inducible Mammalian Expression System

Establishing the ponA-inducible expression system in cultured cells involves the following sequence of procedures:

- Digest the pEGSH vector with the desired restriction enzyme(s) and ligate the insert.
- Transform competent cells with the pEGSH construct.
- Purify the pEGSH construct and determine whether the expression construct can be induced in the cultured cell line by transient cotransfection of the pERV3 and pEGSH vectors.
- Stably transfect cultured cells with the pERV3 vector.
- Isolate G418-resistant stable clones and expand the colonies.
- Examine receptor expression by transient transfection of the pEGSH-Luc vector into G418-resistant clones.
- Stably transfect the receptor-expressing cell line with the pEGSH construct.
- Isolate hygromycin- and G418-resistant stable clones and expand the colonies.
- Induce gene expression with ponasterone A.
- Perform the assay for gene expression.

PROTOCOL FOR THE COMPLETE CONTROL® INDUCIBLE MAMMALIAN EXPRESSION SYSTEM

Digesting the pEGSH Vector and Ligating the Insert

The pEGSH vector contains 11 unique restriction enzymes in the MCS for insertion of the gene of interest (see Figure 3). See reference 6 for protocols covering basic DNA manipulations. Seamless insertion between the *Eam* 1104 I sites can be accomplished using Stratagene's Seamless® cloning kit. The gene of interest to be inserted into the pEGSH vector should contain a Kozak translation initiation sequence.[7] If the gene does not contain a stop codon, Stratagene recommends inserting the gene in reading frame with one of the stop codons found in the *Xba* I (TC<u>TAG</u>A) or *Spe* I (AC<u>TAG</u>T) sites in the MCS or in reading frame with a stop codon located downstream of the sequence of the FLAG epitope.

Stratagene suggests dephosphorylating the digested vector with calf intestinal alkaline phosphatase (CIAP) prior to ligation with the insert DNA. If the vector is digested with more than one restriction enzyme, the small fragment between the two restriction sites (which appears as background) can be removed by electrophoresing the DNA on an agarose gel and recovering the desired vector by electroelution.

After purification and ethanol precipitation of the DNA, resuspend the DNA in TE buffer (see *Preparation of Media and Reagents*). The concentration of the vector DNA should be the same as the concentration of the insert DNA (~0.1 μg/μl).

For ligation, the ideal insert-to-vector ratio is variable; however, a reasonable starting ratio is 2:1 (measured in available picomole ends). The insert-to-vector ratio is calculated as follows:

$$\text{picomole ends / microgram of DNA} = \frac{2 \times 10^6}{\text{number of base pairs} \times 660}$$

Digestion and Ligation Protocol

1. Digest the pEGSH vector with the desired restriction enzyme(s).

2. Prepare the experimental and control samples for ligation by combining the components specified in the following table in microcentrifuge tubes:

Samples (1 and 2) and controls (3–5)	Experimental samples		Controls		
	Insert:vector (1)	Insert:vector (2)	CIAP (3)	Background (4)	Insert purity (5)
Prepared vector (0.1 μg/μl)	1 μl	1 μl	1 μl	1 μl	0 μl
Prepared insert (0.1 μg/μl)	X μl	X μl	0 μl	0 μl	1 μl
10 mM rATP (pH 7.0)	1 μl	1 μl	1 μl	1 μl	1 μl
10× Ligase buffer[a]	1 μl	1 μl	1 μl	1 μl	1 μl
T4 DNA ligase	0.5 μl	0.5 μl	0.5 μl	0 μl	0.5 μl
ddH_2O to 10 μl	X μl	X μl	6.5 μl	7.0 μl	6.5 μl
Expected results	Many colonies	Many colonies	Few colonies	No colonies	No colonies

[a] See *Preparation of Media and Reagents.*

3. Incubate the tubes overnight at 4°C. When performing a blunt end ligation, incubate the tubes overnight at 12–14°C.

Transforming Competent Cells with the pEGSH Construct

1. Transform competent cells with 1–2 μl of the ligation mixture.

2. Plate the transformation on LB–ampicillin agar plates (see *Preparation of Media and Reagents*).

Small-scale DNA preps of the transformants can be screened for the gene of interest by restriction digestion or PCR. The provided pEGSH forward sequencing primer and T3 promoter sequencing primer can be used to sequence the 5′ and 3′ junctions of the inserted DNA, respectively (see Table I).

TABLE I

Primer Sequences and Positions in the pEGSH Vector

Sequencing primer	Sequence	Position (bp)
pEGSH forward sequencing primer	5′-CTCTGAATACTTTCAAAAGTTAC-3′	4639–4661
T3 promoter sequencing primer	5′-AATTAACCCTCACTAAAGGG-3′	4822–4803

Determining Whether the Expression Construct Can Be Induced in the Cultured Cell Line

Note *Stratagene recommends cesium chloride-banded DNA preps (or a comparable protocol) of positive transformants before transfection into cultured cells.*

The cultured cell line chosen for expression of the gene of interest can be transiently cotransfected with the pERV3 receptor vector and the pEGSH construct (containing the gene of interest) as a quick method to determine whether or not the expression construct can be induced in the cell line. The preferred method of transfection depends on the chosen cultured cell line. See Stratagene's Web site at *www.stratagene.com* for information on Stratagene's calcium phosphate- and liposome-mediated transfection kits. Stratagene recommends transfecting the cells at least in duplicate to enable a comparison of uninduced and induced expression levels.

Notes *Stratagene recommends transfecting cells with several different amounts of DNA in order to determine the optimal concentration. The optimal amount of DNA will vary between cell lines and transfection methods. Follow the manufacturer's transfection protocol. Expression of the inserted gene can be induced by adding 1–10 μM ponA to the medium 4–20 hours before harvesting the cells. Parameters will need to be optimized for each particular system.*

Results from transient assays should be used to ascertain whether the gene of interest can be induced in the cell line of choice and to determine the maximal expression levels that can be expected in stable cells or transgenic animals. Uninduced pEGSH background expression will be considerably higher in transient transfections than stable transfections. This background is due to the high copy number of the plasmid and accessibility of the free nuclear DNA to core transcription factors. In stably transfected cells, however, the pEGSH vector is integrated into the host chromosome and is naturally repressed.

Stably Transfecting Cultured Cells with the pERV3 Vector

Perform a stable transfection of cultured mammalian cells with the pERV3 receptor vector using an appropriate method for the chosen cell line. Stratagene recommends calcium phosphate-based transfections for stable transfection. Calcium phosphate-based transfections require 5–15 μg of DNA/100-mm culture plate.

Use of Antibiotics for Cell Selection

Not all mammalian cell lines are equally sensitive to the antibiotics G418 and hygromycin. The minimal lethal concentration can range from 100 μg/ml to 1 mg/ml. The antibiotic concentration to be used for selection must be determined for **each** cell line **before** beginning the experiment.

Consult the available literature on the sensitivity of cell lines to G418 and hygromycin. If no information about the sensitivity of a particular cell line is available, a simple way to determine sensitivity is to grow cultures in a multiwell plate with a range of antibiotic concentrations between the individual wells. The optimal concentration is the lowest one that kills all of the cells within 10–14 days. (Rapidly dividing cells may be killed more readily since the antibiotic appears to act mainly on dividing cells.)

In some cases, it may be possible to reduce the concentration of the antibiotic after initial selection and still maintain selective pressure for the marker gene. For example, NIH3T3 cells are generally selected in 400 μg/ml G418, but selective pressure for the neomycin-resistance (Neo^r) gene can be maintained in 250 μg/ml.

Isolating G418-Resistant Stable Clones and Expanding the Colonies

1. Select for transfected cells by adding G418 (100 μg/ml–1 mg/ml, depending on the cell line) to the medium.

2. Isolate individual clones and expand the resulting colonies.

Examining Receptor Expression

Examine the expanded clones for expression of the VgEcR and RXR receptors by transiently transfecting the G418-resistant clones with the pEGSH-Luc vector and inducing luciferase transcription with ponA.

Note *Stratagene recommends examining at least 20 individual clones, as the site of integration of the plasmid into the chromosome will affect how well the receptors are expressed. For most cell types, relatively small quantities of pEGSH-Luc (10–100 ng/10^5 cells plated) should give induction ratios of ≥20-fold and reach ≥10^5 RLUs when fully induced in these assays.*

Prescreened stable cell lines that express the VgEcR and RXR receptors at optimal levels are available from Stratagene.

Stably Transfecting the Receptor-Expressing Cell Line with the pEGSH Construct

Once the luciferase assay confirms that the VgEcR and RXR receptors are being expressed, the cell line from *Examination of Receptor Expression* can be stably transfected with the pEGSH vector containing the gene of interest. Use an appropriate method of stable transfection for the chosen cell line.

Isolating Hygromycin- and G418-Resistant Stable Clones and Expanding the Colonies

1. Apply selective pressure to the cells by adding hygromycin to the medium (100 μg/ml–1 mg/ml, depending on the cell line). G418 in the medium maintains selective pressure for the pERV3 vector.

2. Isolate individual clones and expand the resulting colonies.

Note *Stratagene recommends isolating at least 20 individual clones, as expression is insertion-site dependent.*

Inducing Gene Expression with Ponasterone A

Once expression of the pERV3 vector and the pEGSH construct containing the gene of interest has been confirmed, expression of the gene of interest can be induced by adding ponA to the medium.

1. Split each sample of stably transfected cells into at least two 35–100-mm culture dishes at a density that will allow the cells to reach 70–80% confluence by the end of the induction period (seeding density will depend on the chosen cell type).

2. Add ponA to a final concentration of 1–10 μM (diluted from a 1 mM stock, see *Preparation of Media and Reagents*) to one of the plates. Add an equivalent volume of solvent (ethanol) to the uninduced sample plates.

3. Incubate the cells at 37°C in a CO_2 incubator for 4–20 hours. The optimal induction time and ponA concentration will depend on the chosen cell type and gene of interest.

4. Wash the cells with 1× PBS and harvest them using a rubber policeman. Examine the cells for expression of the desired gene product by comparing the induced plates to the uninduced plates. Use a quantitative protein assay to determine total protein levels in each harvested sample. Normalize the protein levels before comparing expression levels.

Note *The level of expression can depend on many variables including the gene of interest, cultured cell line, and method of detection. Parameters for each system must be optimized in order to maximize expression levels.*

Performing the Assay for Gene Expression

It is possible to monitor expression of the gene of interest at the level of protein expression or RNA expression. To directly detect the protein of interest, the FLAG epitope can be fused to the C-terminal of the protein of interest, and the expressed protein can be detected by immunoscreening with the M2 α-FLAG antibody. RNA levels can be monitored using antisense RNA probes transcribed from the T3 promoter.

TROUBLESHOOTING

Observation	Suggestion
Transfection efficiencies are low	DNA is not pure. Purify the DNA by cesium chloride banding
	DNA concentration is not optimal. In cotransfections, optimize vector quantities for the chosen cell line
	Antibiotic concentration is not optimal. In stable transfections, perform an antibiotic kill curve on the chosen cell line
Induction of gene expression is low	PonA concentration is not optimal. Optimize the concentration and exposure time of ponA for the chosen cell line
	Integration site of both the pERV3 vector and the pEGSH vector affects expression. Isolate and test at least 20 individual stable clones. Produce pERV3 and pEGSH double-stable cell lines by sequential selection of an optimal pERV3 line. Use this selected line to produce the double-stable line
	Cells have not adequately recovered from transfection. Lengthen the time of recovery from the transfection
	Total protein levels of repressed and induced samples are not normalized. Protein levels should be identical when comparing expression levels
	Expression of the inserted gene is not optimal in the chosen cell line. Confirm that the chosen cell line can support expression of the gene product
	Receptor expression is not compatible with the chosen cell line. Confirm that the CMV promoter is functional in the chosen cell line using a CMV reporter plasmid
	Cell density is too high at the time of induction. Cell density should be ≤80% at the time of induction

Preparation of Media and Reagents

LB Agar (per Liter) 10 g of NaCl 10 g of tryptone 5 g of yeast extract 20 g of agar Add dH_2O to a final volume of 1 liter Adjust pH to 7.0 with 5 N NaOH Autoclave Pour into petri dishes (~25 ml/100-mm plate)	**LB–Ampicillin Agar (per Liter)** Prepare 1 liter of LB agar Autoclave Cool to 55°C Add 5 ml of 10-mg/ml-filter-sterilized ampicillin Pour into petri dishes (~25 ml/100-mm plate)
10× Ligase Buffer 500 mM Tris-HCl (pH 7.5) 70 mM $MgCl_2$ 10 mM dithiothreitol (DTT) **Note** *rATP is added separately in the ligation reaction.*	**LB Liquid Medium (per Liter)** 10 g of NaCl 10 g of tryptone 5 g of yeast extract Add dH_2O to a final volume of 1 liter Adjust to pH 7.0 with 5 N NaOH Autoclave
1× Phosphate-Buffered Saline (PBS) 137 mM NaCl 2.6 mM KCl 10 mM Na_2HPO_4 1.8 mM KH_2PO_4 Adjust the pH to 7.4 with HCl	**1 mM Ponasterone A** Resuspend 1 mg of ponasterone A in 2 ml of 100% (v/v) ethanol Store at –20°C **TE Buffer** 5 mM Tris-HCl (pH 7.5) 0.1 mM EDTA

REFERENCES

1. No, D., Yao, T. P. and Evans, R. M. (1996) *Proc Natl Acad Sci U S A* 93(8):3346-51.
2. Wyborski, D. L. and Vaillancourt, P. (1999) *Strategies* 12(1):1-4.
3. Chen, J. D., Umesono, K. and Evans, R. M. (1996) *Proc Natl Acad Sci U S A* 93(15):7567-71.
4. Padgett, K. A. and Sorge, J. A. (1996) *Gene* 168(1):31-5.
5. Hopp, T., Prickett, K., Price, V., Libby, R., March, C. *et al.* (1988) *BioTechnology* 6:1204-1210.
6. Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
7. Kozak, M. (1999) *Gene* 234(2):187-208.

ENDNOTES

Complete Control® Inducible Mammalian Expression System

Catalog #271460, #217461, and #217468

QUICK-REFERENCE PROTOCOL

- Digest the pEGSH vector with the desired restriction enzyme(s) and ligate the insert
- Transform competent cells with the pEGSH construct
- Purify the pEGSH construct
- Determine whether the expression construct can be induced in the cultured cell line by transient cotransfection of the pERV3 and pEGSH vectors
- Stably transfect cultured cells with the pERV3 vector
- Isolate G418-resistant stable clones and expand the colonies
- Examine receptor expression by transient transfection of the pEGSH-Luc vector into G418-resistant clones
- Stably transfect the receptor-expressing cell line with the pEGSH construct
- Isolate hygromycin- and G418-resistant stable clones and expand the colonies
- Induce gene expression with ponasterone A
- Perform the assay for gene expression

(2000/03) Stratagene develops and manufactures biological products and instruments designed to improve the speed and accuracy of molecular biology and genomics research. We market our products to researchers at academic and government institutions and pharmaceutical, biotechnology and industrial companies, in the

U.S. and internationally. These researchers use our products to identify genes, study how cells are regulated by genes, determine the molecular mechanisms of health and disease, search for new drug therapies and test the safety of compounds used in food and pharmaceuticals. Our 1999 revenues were approximately $51 million.

We have been serving the molecular biology research market since 1984. Our products incorporate a diverse range of molecular biology technologies, such as gene transfer, gene expression, gene cloning, gene libraries, functional genomics, nucleic acid purification and analysis, microarrays, DNA replication and DNA sequencing.

Keywords

Differential Display, Antibodies, Gene Expression, Immunoassays, Human Primary Cells, Reactive Oxygen Species, Adipocytes, In Situ Hybridization, expression, Competent Cells, Packaging Extracts, Electrophoresis, Gene Targeting, Instruments, Labware, RoboCycler®, Electroporation, StrataCooler®, Immunoscreening Kits, microarrays, Restriction Enzymes, DNA Binding Protein, DNA Polymerase, Kinases, Ligases, Nucleases, PCR Enhancers, Phosphatase, Transferase, Restriction Enzymes, Reverse Transcriptase, RNA Polymerase, Thermostable Polymerase, Molecular Medicine, hNT Neurons, cDNA Libraries, Gene Mapping, Nucleic Acid, Translation Kit, Markers, RNA Capping, Kits, RNase Inhibitors, RNA Transcription, PCR Analysis, Molecular Beacons, RNA, Signal Transduction, Antibodies, Pathway, Protein Kinase, Assays, Substrates, Synthetic Oligonucleotides, Primers, Apoptosis, Big Blue, Data Analysis, DNA Sequencing, Micronucleus, Molecular Toxicology, Mutagenesis Testing, Reactive Oxygen Species, Transgene, Transfection, Mycoplasma, Detection, Cloning, Vectors, Expression, drug discovery, genetic toxicology, portal, data sources, Discovery Clone Collection, 25,000 unique clones

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Complete Control® Inducible Mammalian Expression System

- Dose-responsive inducible mammalian expression
- Induction as high as 1,700 fold
- Tightly repressed
- Novel IRES site allows expression of both receptor subunits from the same CMV promoter
- Retroviral and plasmid gene delivery systems available
- Stable receptor cell lines available

Applications

- Control expression of heterologous gene products in mammalian cells
- Retroviral system available for gene delivery to difficult-to-transfect cells

Promoter:

CMV promoter in pERV3 plasmid and pFB-ERV retroviral vector. SP1/minimal promoter in pEGSH plasmid and pCFB-EGSH retroviral vector.

Selection

pERV plasmid: G418 in mammalian cells, Kanamycin in *E. coli*
pEGSH plasmid: Hygromycin in mammalian cells, Ampicillin in *E. coli*
pFB-ERV retroviral vector: G418 in mammalian cells and Kanamycin or Ampicilin in *E. coli*
pCFB-EGSH retroviral vector: Hygromycin in mammalian cells and Ampicillin in *E. coli*

Tight Control

The Complete Control® Inducible Mammalian Expression System is designed to provide the tightest control of expression available in mammalian cells. The system is based on a synthetic ecdysone-inducible receptor and a synthetic receptor recognition element that modulates expression of your gene of interest. The artificial nature of the receptor and the recognition element ensures that endogenous host transcription factors and genes will not be activated, providing extremely low background in the system. In addition, the receptors are bound to the recognition element in the absence of inducer, further reducing background expression. After addition of the inducer, ponasterone A, a conformational change of the receptor subunits removes transcription repressors and recruits transcriptional machinery to activate transcription.

Powerful Induction

Induction is fast and potent, in just 20 hours we have observed as high as 1,747-fold induction in transient assays and 1,030-fold induction in a double-stable cell line. Using a range of ponasterone A gives a linear dose response curve, allowing the amount of induction to be easily moderated.

Innovative Vectors

Two vectors are used to control expression in the Complete Control system. The synthetic receptor is constitutively expressed as a heterodimer consisting of the ecdysone receptor (EcR) and the retinoid-X-receptor (RXR). Both subunits of the receptor are produced from the pERV3 vector, using an internal ribosomal re-entry site (IRES) to allow expression of both proteins from the same CMV promoter. This unique design allows expression of the heterodimer receptor in a wide variety of cell lines. The expression vector, pEGSH, is designed for easy detection of expressed protein by either RNase protection assay using T3 antisense probes or by western analysis using the FLAG® epitope.

We have developed a retroviral version of the Complete Control Inducible Mammalian Expression System to expand its utility. Gene delivery using retroviruses often yields transduction efficiencies close to 100%, and the proviral copy number can be easily controlled by varying the multiplicity of infection (MOI). This latter feature is particularly important for inducible systems, for which low basal expression and high induction ratios are affected by copy number. Thus, transduction of the target cell with virus at an optimal MOI should yield a high frequency of clones capable of mediating desirable expression profiles without exhaustive colony screening. The two retroviral vectors are pFB-ERV, an MMLV-based replication-defective retroviral vector that delivers the ecdysone receptor proteins RXR and VgEcR, and the pCFB-EGSH retroviral vector containing the inducible cassette. Used together, we have attained induction ratios of >1,000-fold in tissue culture cells.

Save Time with Stable Cell Lines

The best method to create double-stable cell lines is sequentially, first establishing pERV3 stable cell lines and selecting the line that gives the highest induction and the lowest backgrounds using the pEGSH-luc control vector in transient assays. Then, use this line to create a double-stable cell line using the pEGSH expression construct containing your gene of interest. Strategene has eliminated the first step of this process with a collection of pERV3 stable cell lines, derived from CHO, NIH3T3 and 293 cells. Each cell line is tested for viability and functionally tested in a transient assay using the pEGSH-luc control vector and monitoring luciferase expression after induction with ponasterone A.

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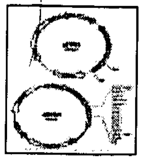
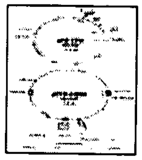
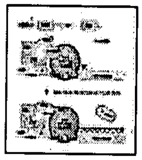


Complete Control® Vector Maps



Complete Control® Retroviral Vector Maps



The Ecdysone-Inducible Expression System
The Nuclear receptor proteins RXR and VgEcR are coexpressed from the CMV promoter VgEcR: hybrid nuclear receptor comprised of the ecdysone receptor (EcR) ligand-binding and dimerization domains, the VP16 transcriptional activation domain and the glucocorticoid receptor (GR) DNA binding domain. The heterodimeric ecdysone receptor remains bound to five copies of the E/GRE recognition element located upstream of a minimal promoter in the inducible expression cassete. The inductible promoter remains transcriptionally silent until induction with the ecdysone analogs muristerone A or ponasterone A. Interaction between the inducer and the EcR ligand-binding domain results in the recruitment of coacivator(s) and, thus, transcriptional activation that can reach over three orders of magnitude.

Vector Details

pERV3

Sequence	Restriction Site	View Map

pEGSH

Sequence	Restriction Site	View Map

pFB-ERV

Sequence	Restriction Site	View Map

pCFB-EGSH

Sequence	Restriction Site	View Map

pCFB-EGSH-Luc

View Map

Amount	Catalog #	Price	
Complete Control® Vector Kit pERV3 vector, pEGSH vector, 1 mg ponasterone A, Sequencing primers			
1 kit	217468	545.00	add
pEGSH Vector Ecdysone-inducible vector, MCS contains restriction sites positioned for directional cloning of inserts derived from Lambda ZAP -derived cDNA vectors, Lambda gt10, Lambda gt11, HybridZAP vectors and other two-hybrid libraries, Monitor gene expression using ?-FLAG immunoprecipitation or T3 antisense RNA probes			
20 µg vector, Sequencing primers	217461	260.00	add
pERV3 Vector Receptor expression vector, CMV promoter easily replaced to confer cell-type specificity to receptor expression			
20 µg vector, Sequencing primers	217460	260.00	add
pFB-ERV Retroviral Vector Receptor expression vector, CMV promoter easily replaced to confer cell-type specificity to receptor expression			
20 µg vector, 20 µg pCFB-EGSH-Luc control vector	217564	260.00	add
pCFB-EGSH Retroviral Vector			
20 µg vector, 20 µg pCFB-EGSH-Luc control vector	240028	260.00	add

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Manuals

217460 Complete Control® pERV3 Vector
217461 Complete Control® pEGSH Vector
217468 Complete Control® Vector Kit
217564 Complete Control® pFB-ERV Retroviral Vector
240028 Complete Control® pCFB-EGSH Retroviral Vector

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WARF language:±±Use of these vectors is covered by U.S. Patent No. 4,937,190 and is limited to use solely for research purposes. Any other use of the pERV3 vector requires a license from WARF.

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1. Complete Control system for inducible mammalian expression
   ... is a member of the **rxr** heterodimer family of nuclear receptors. in ...
   ... - x-receptor (**rxr**), the mammalian homologue of usp. the ecr-**rxr** ...
   ... to dimerize with **rxr** and trans -activate in a pona-dependent manner ...
   ... , or by natural **rxr** heterodimer family receptors, which require ...
   33.2k

2. pFB-ERV: Retroviral Delivery of the Ecdysone Receptor Proteins
   ... receptor proteins **rxr** and vgecr. in stable cell lines infected with ...
   ... receptor proteins **rxr** and vgecr. vector description the vector pfb- ...
   ... proteins vgecr and **rxr** are expressed from the first and second open ...
   ... translation of the **rxr** and neo orfs is mediated by the emcv-ires (both ...
   28.3k

3. Complete Control® Inducible Mammalian Expression System
   ... -x-receptor (**rxr**). both subunits of the receptor are produced ...
   ... receptor proteins **rxr** and vgecr, and the pcfb-egsh retroviral vector ...
   ... receptor proteins **rxr** and vgecr are coexpressed from the cmv promoter ...
   30.8k

4. Strategies 14.1 - p. 15-16
   ... receptor proteins **rxr** and vgecr, and the pcfb-egsh retroviral vector (...
   ... the vgecr and **rxr** ecdysone receptor proteins. two days following ...
   31.8k

rxr GO

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pFB-ERV: Retroviral Delivery of the Ecdysone Receptor Proteins

Peter Vaillancourt • Katherine A. Felts
Stratagene

We describe the vector pFB-ERV, an MMLV-based replication-defective retroviral vector for delivery of the ecdysone receptor proteins RXR and VgEcR. In stable cell lines infected with an estimated single copy of the receptor cassette, induction ratios of greater than 200-fold are attainable in transient reporter transfection assays. The neo-resistance marker is expressed as the third open reading frame (ORF) in a tricistronic CMV expression cassette (the two receptors comprise the first and second ORFs); cell lines harboring single-copy integrants are resistant to as high as 1 mg/ml G418. The CMV promoter[###] *is flanked by unique restriction sites and, thus, can be replaced with a cell-type specific promoter of interest.*

DNA vector-based systems that allow precise control of gene expression in vivo have become invaluable for the study of gene function in a variety of organisms, particularly when applied to the study of developmental and other biological processes for which the timing or dosage of gene expression is critical to gene function. Such systems have also been successfully used to overexpress toxic or disease-causing genes, to induce gene targeting, and to express antisense RNA. Inducible systems are currently being used by pharmaceutical companies to facilitate screening for inhibitors of clinically relevant biological pathways, and potential applications for gene therapy are being explored.[1]

Stratagene's complete control® inducible system[±] is based on the insect molting hormone ecdysone, which can stimulate transcriptional activation in mammalian cells harboring the ecdysone receptor protein from *Drosophila melanogaster*.[2,3] The ecdysone-inducible system has a number of advantages over alternative systems. Firstly, the lipophilic nature and short in vivo half-life of the ecdysone analog ponasterone A (ponA) allow efficient penetrance into all tissues including brain, resulting in rapid and potent inductions and rapid clearance. Secondly, ecdysteroids are not known, nor are they expected, to affect mammalian physiology in any measurable way. Thirdly, the heterodimeric ponA responsive receptor and receptor DNA recognition element have been genetically altered such that *trans*-activation of endogenous genes by the ecdysone receptor, or of the ponA-responsive expression cassette by endogenuos transcription factors, is extremely unlikely.

In addition, it has been found that in the absence of inducer the heterodimer remains bound at the promoter in a complex with corepressors and histone deacetylase, and is, thus, tightly repressed until ligand binding, at which time high-level transcriptional activation occurs (i.e., the heterodimer is converted from a tight repressor to a *trans*-activator). Using the Complete Control plasmid-based system, induction ratios of greater than 1,000-fold have been achieved in both transient transfections and in stable cell lines.[3]

Using plasmid-based vectors for controlled gene expression is limiting because many cell types that are of academic, industrial, or clinical interest are difficult or virtually impossible to transfect using current transfection methods. In particular, primary human cell lines and lymphocytes are best transduced using viral delivery systems. The most popular and user-friendly of these are the retroviral vectors.[4,5] Infection with retroviruses often yields transduction efficiencies close to 100%, and the proviral copy number can be easily controlled by varying the multiplicity of infection (MOI). This latter feature is particularly important for inducible systems for which low basal expression and high induction ratios are affected by copy number. Hence, viral infection (at an optimal

MOI) of the target cell should yield a high frequency of clones capable of mediating desirable expression profiles without exhaustive colony screening.

We describe the vector pFB-ERV, an MMLV-based replication-defective retroviral vector for delivery of the ecdysone receptor proteins RXR and VgEcR.

Vector Description

The vector pFB-ERV contains a tricistronic message transcribed from the CMV promoter (Figure 1). The receptor proteins VgEcR and RXR are expressed from the first and second open reading frames (ORF), respectively,

and the neomycin-resistance gene is expressed from the third ORF. Translation of the RXR and Neo ORFs is mediated by the EMCV-IRES (both IRESs are identical). In this context, G418 selection and maintenance of the expression cassette in stably infected cells ensures that the receptor-encoding mRNA is transcribed. The CMV expression cassette was built into a self-inactivating (SIN) vector backbone,[6,7] in which the retroviral promoter within the U3 region of the 3¢ LTR was deleted. In SIN vectors, viral genomic RNA is expressed from the 5¢ viral LTR in packaging cells; however, upon infection the virus replicates in such a way that the (inactive) 3¢ U3 promoter sequences are transferred to the 5¢ LTR, and the proviral 5¢ promoter is then inactive in infected cells. The rationale for this construction is the following: The CMV promoter is stronger than the MMLV LTR and is persistently active in a wider range of cell types, and inactivation of the 5¢ LTR obviates potential interference with the CMV promoter; the CMV promoter can be readily replaced with a cell-type promoter of interest using the unique *Eco*R I and *Fse* I sites without concern of ubiquitous read-through from the 5¢ LTR; spurious activation/transcription of 3¢-flanking endogenous genes from the promoter within the 3¢ LTR will not occur in the SIN vector; and inactivation of the proviral LTRs protects against mobilization of proviral pFB-ERV derivatives by endogenous retroviral structural proteins.

Titer Determination

Vector titer was determined by G418-resistant colony formation. Amphotropic virus was produced by transient transfection using the producer line HW293-A (unpublished data), and viral supernatants were used to infect NIH3T3 cells. In Table 1, the titers for both experiments are on the order of 10^5 colony forming units (cfu)/ml. Even at high dilutions of supernatant ($1:10^3$), which likely give rise to single-copy infected cells in accordance with the Poisson kinetics of viral transduction, colonies are resistant to as high as 1 mg/ml G418 without substantial loss of titer, indicating efficient expression of the Neo gene from the third ORF.

Performance of pFB-ERV in Mass Populations of Infected Cells

NIH3T3 cells were infected with various dilutions of viral supernatant, and one day following infection the cells were transfected with the ecdysone-inducible reporter vector pEGSH-luc. The following day, cells were induced for 20 hours with ponA, then assayed for luciferase activity. In Figure 2 (at an MOI of 1.0), a strong induction is achieved, which is reproducible in separate infected populations.

Analysis of Clonal Isolates of Individual Cells Infected with pFB-ERV

NIH3T3 cells were infected with pFB-ERV supernatants and selected with 600 µg/ml G418. Resistant colonies were picked and expanded. In an initial screen, 24 colonies were transfected with reporter vector, and induced with 10 µM ponA or an equivalent volume of vehicle. All 24 of the infected lines showed a pon A- dependent induction to some degree (Figure 3). One clone, A610-20 (clone #20, Figure 3) gave an induction of greater than 200-fold in the initial screen. A retest of this cell line gave an induction of approximately 250-fold (Figure 3B). This clone was produced by infection at an MOI of 0.1, and, therefore, the colony theoretically has a single integrated copy of the receptor expression cassette.

Conclusions

The 200-fold induction seen by transient reporter transfection of the A610-20 receptor line is likely to be an under-representation of the induction profile to be expected in double-stable lines made by selecting stably integrated inducible vectors. This is due to

the tight repression that occurs for chromosomally integrated ecdysone-responsive promoters, compared with the relatively high background normally seen in transient transfection assays in which the reporter is free in the nucleus. The stable cell line ER-CHO, which was made using the receptor-expressing plasmid pERV3, shows at best 50-fold induction ratios by transient reporter transfection but the derivative double-stable line consistently gives induction ratios of 700- greater than 1,000-fold.[3] Accordingly, we expect that the A610-20 receptor line will give rise to double-stable lines with induction ratios well in excess of 1,000-fold.

Retroviral delivery of the ecdysone receptors should significantly decrease the time and labor required to screen for stable clones that express the receptors at optimal levels, particularly for cell types that are normally difficult to transfect. However, for difficult-to-transfect cell types, delivery of the receptors with the pFB-ERV virus only solves half the problem. Currently, the ecdysone-inducible vectors need to be delivered by transfection. At this writing, several ecdysone-inducible retroviral constructions are being evaluated for efficient delivery of inducible expression cassettes that allow fine control of expression and high-level induction of the gene of interest. The use of a two-virus system for delivery of the edysone-inducible expression system will further expand the range of cell types in which ecdysone-regulated expression can be achieved, while further reducing the time, cost, and labor of screening for clones that show optimal expression characteristics.

Please contact Technical Service for availability

REFERENCES

1. Schockett, P.E. and Schatz, D.G. (1996) *Proc. Natl. Acad. Sci.* 93: 5173-5176.
2. No, D., et. al. (1996) *Proc. Natl. Acad. Sci.* 93: 3346-3351.
3. Wyborski, D. and Vaillancourt, P. (1999) *Strategies in Mol. Biol.* 12(1): 1-4.
4. Miller, A.D. (1997) *In Retroviruses* (Eds., Coffin, J.M., Hughes, S.H., and Varmus, H.E.) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. pp. 437-473.
5. Felts, K.A., et. al. (1999) *Strategies in Molec. Biol.* 12(2): 74-77.
6. Yu, S.-F., et. al. (1986) *Proc. Natl. Acad. Sci.* 83: 3194-3198.
7. Yee, J.-K. (1987) *Proc. Natl. Acad. Sci.* 84: 5197-5201.

###,± See trademarks and patents

Figure 1: Map of pFB-ERV

The vector is derived from pBR322 and includes the Col E1 origin of replication and the ampicillin-resistant gene.

Figure 2: Ecdysone Receptor Activity in Mass Populations of Infected Cells

NIH3T3 cells were infected with cell culture supernatants containing pFB-ERV virus at an MOI of either 0.1 or 1.0, and after 24 hours were transfected with an optimal amount of ecdysone-inducible reporter plasmid pEGSH-luc. The day following transfection, cells were induced for 20 hours with ponasterone A (+)(10 μM) or mock-induced with an equivilant volume of ethanol vehicle (-). Following induction, cells were harvested and assayed for luciferase activity. Each bar represents a separate population of infected/transfected cells.

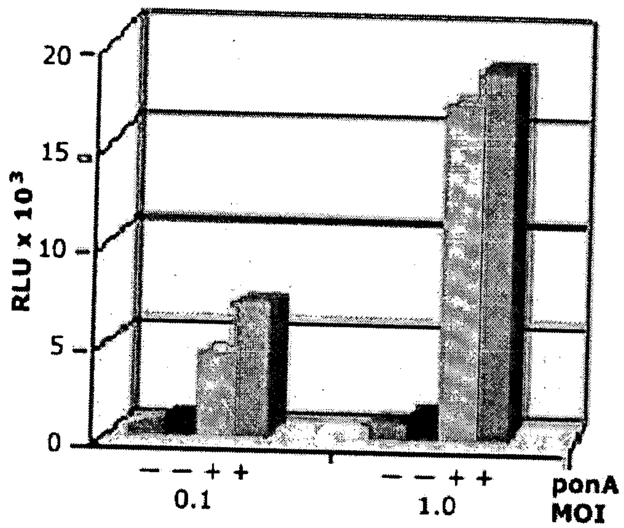


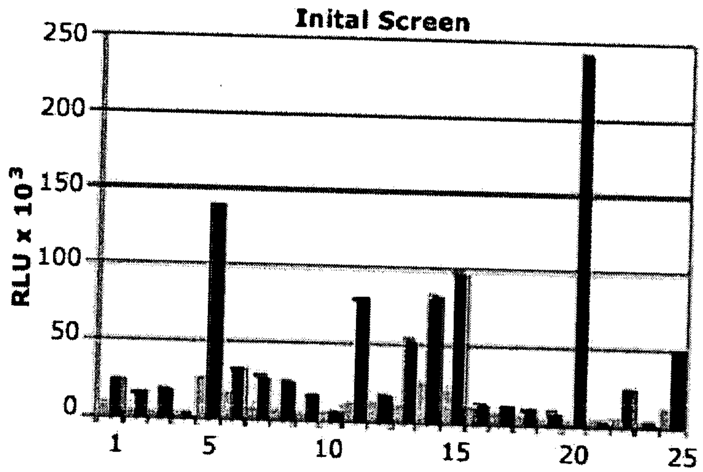
Figure 3: Evaluation of pFB-ERV Stable Cell Lines

Infected NIH3T3 cells were selected with G418 (600 μg/ml) and expanded.

Panel A: Each of 24 individual clonla populations were transfected in duplicate with an optimal amount of pEGSH-luc and induced with ponA (dark bars) or mock-induced with an equivalent amount of ethanol vehicle (light bars), and assayed as described in the legend to Figure 2.

Panel B: Clone number "20" (referred in the text as clone A610-20) was retested and it exhibited an induction ration of over 200-fold.

A



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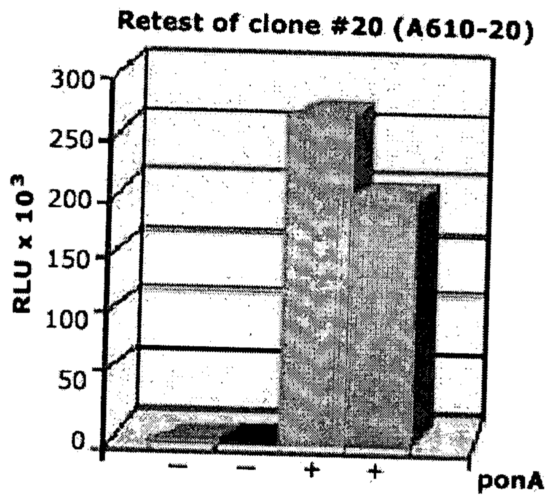

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complete control® system for inducible mammalian expression

Versatile Vectors for Ponasterone A- Inducible Control of Gene Expression in Mammalian Cells

Denise Wyborski • Peter Vaillancourt
Stratagene

The complete control® mammalian expression system ± allows tight control of gene expression in a wide range of mammalian cell types. The inducible promoter used in the system is naturally repressed in the absence of the ecdysone analog ponasterone A (ponA). In a double-stable cell line, a linear dose-response is achieved over a wide range of ponA concentrations (4 fold to >500 fold) and, in a time-course experiment, a linear increase was achieved from 1 hour post induction (5 fold) to 20 hours (1,030 fold). Coexpression of both receptors from a single dicistronic transcript from the vector pERV3 facilitates replacement of the CMV promoter with a cell type-specific promoter of interest. The inducible vector pEGSH is engineered so that expression of the gene of interest can be monitored by Western blot analysis or RNA detection.

DNA vector-based systems that allow precise control of gene expression in vivo are invaluable for studying gene function in a variety of organisms, particularly when studying developmental and other biological processes for which the timing or dosage of gene expression is critical to gene function. Such systems are successfully used to overexpress toxic or disease-causing genes, induce gene targeting, and express antisense RNA. Pharmaceutical companies currently use inducible systems to facilitate screening for inhibitors of clinically relevant biological pathways and to explore potential applications for gene therapy.[1]

Most inducible mammalian systems currently available employ either natural promoters that are induced by heavy-metal ions, heat shock, growth factors, and steroid hormones or employ synthetic promoters and inducible activators that often contain *cis* and *trans* elements derived from bacteria or yeasts.[2] However, the majority of these systems have drawbacks: pleiotropic effects caused by the inducer or the transcriptional activator, prohibitively high background expression in the absence of inducer, or poor penetrance and/or clearance of the inducer in some tissues.

Stratagene has recently introduced a better system for controlled mammalian expression based on the finding that the insect molting hormone, ecdysone, stimulates transcriptional activation in mammalian cells harboring the ecdysone receptor protein from the fruit fly *Drosophila melanogaster.*[3] The ecdysone analogs ponA and muristerone A (murA) efficiently penetrate all tissues, including the brain, due to their lipophilic nature and short in vivo half-life. This results in rapid and potent inductions and rapid clearance. Ecdysteroids are not known to affect mammalian physiology in any measurable way.

System Description

The ecdysone receptor (EcR) is a member of the RXR heterodimer family of nuclear receptors. In mammalian cells, the EcR heterodimerizes with the retinoid- X-receptor (RXR), the mammalian homologue of USP. The EcR-RXR heterodimer is capable of binding to and activating reporters that contain multiple copies of the ecdysone-responsive element (EcRE). However, because EcRE-containing reporters can be specifically *trans*-activated by some other lipophilic steroids, the EcR protein and EcRE recognition sequence were modified to create both a synthetic ecdysone-inducible receptor that would not bind to and *trans*-activate any endogenous host genes, as well as a synthetic recognition site that would not be recognized by host transcription factors.

Three amino acids in the EcR DNA-binding domain (DBD) were mutated to change its

DNA-binding specificity to that for the glucocorticoid receptor (GR).[3] The GR-EcR (GEcR) fusion protein retains the ability to dimerize with RXR and *trans*-activate in a ponA-dependent manner and is able to recognize and activate reporters containing the synthetic binding site AGTGCA N1 TGTTC (E/GRE). This binding site is extremely unlikely to be recognized by steroid family receptors, which require perfect inverted half-sites, or by natural RXR heterodimer family receptors, which require single nucleotide spacing between half-sites. Finally, the GEcR receptor was further modified by replacing the EcR AD with the more potent VP16 *trans*-activator to create the receptor protein VgEcR (Figure 1).

Versatile Vectors

Two vectors are required for ponA-inducible expression of the gene of interest: the receptor expression vector pERV3, from which VgEcR and RXR are constitutively expressed, and the ecdysone-inducible vector pEGSH (Figure 2). The pERV3 vector is engineered such that both receptors are expressed from a single mRNA transcribed from the CMV promoter. We accomplished this by placing the internal ribosome entry site (IRES) from encephalomyocarditis virus (EMCV)[±±] upstream of the second (RXR) open reading frame (ORF), which allows high-level internal (cap-independent) initiation of translation of ORFs positioned downstream in an appropriate context.[4] Expressing both receptors from a single transcript has many advantages. For example, transcription of this expression cassette can be achieved in a wide variety of cell types from a single plasmid, due to the versatility of the CMV promoter; and the CMV promoter in this construct can be readily replaced with other promoters to confer cell-type specificity to receptor expression—an advantage that is particularly attractive for the construction of transgenic animals. The plasmid also contains a neomycin-resistance gene, which is expressed in both *E. coli* (kanamycin-resistance) and mammalian cells (G418-resistance).

The ecdysone-inducible expression vector pEGSH contains the ponA-inducible expression cassette comprised of 5 E/GRE binding sites upstream of a minimal promoter consisting of three SP1 sites, followed by the *D. melanogaster* hsp27 minimal promoter (Figure 2B). The vector contains the hygromycin-resistance gene to allow stable selection in cells transformed with the pERV3 plasmid. The MCS of pEGSH was engineered to contain an array of restriction sites positioned for directional cloning of inserts derived from Lambda ZAP®-derived cDNA vectors*, λgt10, λgt11, HybriZAP® vectors, and other two-hybrid libraries, in addition to most other popular cDNA cloning and expression vectors.

In addition to this versatile array of restriction sites, the pEGSH MCS contains the following features: the ability to monitor expression of the gene of interest, by either α-FLAG® immunodetection[|| ||] or by RNA detection using T3 antisense RNA probes; and the ability to seamlessly fuse the insert to the FLAG epitope and/or the HSP leader by using the seamless® cloning kit** for which 80% cloning efficiencies are routinely achieved.

Transient Expression Assays

To demonstrate the quality of both the receptor expression and ecdysone-inducible vectors, we performed transient expression assays using the reporter pEGSH-luc in which the coding sequence for the firefly luciferase gene is inserted into the MCS of pEGSH. In these experiments, reporter was cotransfected with various amounts of the pERV3 receptor vector, induced with murA, and assayed for luciferase activity. Figure 3 shows that, in CHO cells, optimal expression is achieved with a relatively small amount of receptor vector (8 ng) to give an induction ratio of over three orders of magnitude. We achieved comparable results for similar experiments carried out in NIH3T3, 293, and CV-1 cells (data not shown).

Production of Stable Cell Lines

The best method to engineer double-stable cell lines is to first produce stable receptor-expressing cell lines using the plasmid pERV3 and then screen stable receptor-expressing lines by transient transfection of the inducible reporter pEGSH-luc to find one that mediates the highest level of ponA-dependent *trans*-activation. Once produced, this line can be used for constructing pEGSH-derived lines. To expedite this process, we have produced three engineered cell lines derived from CHO, NIH3T3, and 293 cells, respectively, in which optimal levels of the receptors are stably expressed. These three lines show high-level inducer-dependent activation in transient expression assays using the pEGSH-luc reporter (Figure 4).

Production of Double-Stable Cell Lines

The stable receptor line ER-CHO (Figure 4A) was stably transfected with the plasmid pEGSH-luc, and hygromycin-resistant clones were screened for ponA-dependent induction of luciferase activity. Figure 5 shows the results for this double-stable cell line designated HSL-34. The results in Figure 5A show that both murA and ponA induce luciferase activity in HSL-34 cells to comparable levels over a 100-fold range of inducer concentration. To more accurately assess the sensitivity of the system, both ponA concentration and induction time were varied. As the results in Figure 5B indicate, a linear response was observed from 80 nM ponA (four fold: the lowest concentration tested) to 10 μM.

To more directly assess the control of protein expression in this cell line, we performed a Western blot. Lysates from uninduced cells and cells induced with increasing amounts of ponA were fractionated by SDS-PAGE, blotted, and probed with α-luciferase polyclonal antisera (Figure 5C). No significant detectable luciferase is expressed in the uninduced extract, whereas a linear increase in signal is observed from 300 nM to 5 μM ponA. In a time-course experiment, a five-fold induction was achieved only 1 hour after adding 10 μM ponA, and a linear increase was observed for up to 20 hours, at which time an induction ratio of 1,030-fold was observed (Figure 5D). We also observed these induction kinetics in transient assays (data not shown).

Taken together, these results indicate that, by varying the ponA concentration, precise control of gene dosage can be achieved with this system. Furthermore, comparatively brief induction periods can result in low to moderate levels of gene expression.

Detection of PonA-Induced Gene Expression

Current commercially available systems lack a convenient tool to monitor the induced expression of the protein of interest when specific antibody is unavailable. To this end, the pEGSH vector has been engineered to provide a convenient means to detect both RNA and protein expression. For RNA detection, the T3 promoter has been positioned downstream of the MCS in the antisense orientation relative to the inducible expression cassette (Figure 2B); labeled antisense RNA can be readily produced in vitro to use as a probe in Northern or dot blots, in situ hybridization, or RNase protection (data not shown).

To directly detect protein expression without the need for protein-specific antibody, the eight amino acid FLAG tag is positioned for C-terminal fusion to the protein of interest. Therefore, the induction of FLAG fusion proteins can be monitored by Western blotting or other immunodetection methods using the α-FLAG M2 antibody (data not shown).

Conclusions

The Complete Control system has several advantages over other inducible systems. The inducer exhibits no pleiotropic effects on cellular physiology and the inducer's lipophilic nature and short in vivo half-life ensure that it rapidly penetrates and clears all tissues, as well as exhibits dose-dependent control of gene expression. In addition, the ecdysone receptor and its DNA recognition element are genetically altered so there is no cross-talk between the system and endogenous pathways. The uninduced basal activity of the system is extremely low and can be induced to over three orders of magnitude. Finally, the system's pERV3 and pEGSH vectors achieve more consistent expression of both receptors in a wide range of cell types, provide convenient means to monitor gene expression, and allow high-efficiency directional cloning of any insert due to the versatile MCS.

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PRODUCT LINK

- **complete control® Inducible Mammalian Expression System**

REFERENCES

1. Schockett, P.E. and Schatz, D.G. (1996) *Proc. Natl. Acad. Sci.* 93: 5173-5176.

2. Deuschle, U., et. al. (1995) *Molec. Cell Biol.* 15(4): 1907-1914.
3. No, D., et. al. (1996) *Proc. Natl. Acad. Sci.* 93: 3346-3351.
4. Borman, A.M. (1997) *Nuc. Acid Res.* 25(5): 925-932.
5. Aslandis, C. and de Jong, P.J. (1990) *Nuc. Acid Res.* 18: 6069-6074.

* U.S. Patent Nos. 5,128,256, 5,286,636 and European Patent No. 286200
* * Patent pending
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